

Thermostable enzyme promoting the fidelity of thermostable DNA polymerases – for improvement of nucleic acid synthesis and amplification *in vitro*

The present invention is related to the field of molecular biology, and more particular, to polynucleotide synthesis. The present invention also relates to a substantially pure thermostable exonuclease, the cloning and expression of a thermostable exonuclease III in *E.coli*, and its use in amplification reactions. The invention facilitates the high fidelity amplification of DNA under conditions which allow decontamination from carry over and the synthesis of long products. The invention may be used for a variety of industrial, medical and forensic purposes.

In vitro nucleic acid synthesis is routinely performed with DNA polymerases with or without additional polypeptides. DNA polymerases are a family of enzymes involved in DNA replication and repair. Extensive research has been conducted on the isolation of DNA polymerases from mesophilic microorganisms such as *E.coli*. See, for example, Bessman et al. (1957) *J. Biol. Chem.* 223:171-177, and Buttin and Kornberg, (1966) *J. Biol. Chem.* 241:5419-5427.

Research has also been conducted on the isolation and purification of DNA polymerases from thermophiles, such as *Thermus aquaticus*. Chien, A. et al., (1976) *J. Bacteriol.* 127:1550-1557 discloses the isolation and purification of a DNA polymerase with a temperature optimum of 80°C from *Thermus aquaticus* YT1 strain. United States Patent No. 4,889,818 discloses a purified thermostable DNA polymerase from *T. aquaticus*, Taq polymerase, having a molecular weight of about 86,000 to 90,000 daltons. In addition, European Patent Application 0 258 017 discloses Taq polymerase as the preferred enzyme for use in the PCR process.

Research has indicated that while Taq DNA polymerase has a 5'-3' polymerase-dependent exonuclease function, Taq DNA polymerase does not possess a 3'-5' exonuclease III function (Lawyer, F.C. et al., (1989) *J. Biol. Chem.*, 264:6427-6437; Bernad A., et al. (1989) *Cell* 59:219). The 3'-5' exonuclease activity of DNA polymerases is commonly referred to as „proofreading activity“. The 3'-5' exonuclease activity removes bases which are mismatched at the 3' end of a

primer-template duplex. The presence of 3'-5' exonuclease activity may be advantageous as it leads to an increase in fidelity of replication of nucleic acid strands and to the elongation of prematurely terminated products. As *Taq* DNA polymerase is not able to remove mismatched primer ends it is prone to base incorporation errors, making its use in certain applications undesirable. For example, attempting to clone an amplified gene is problematic since any one copy of the gene may contain an error due to a random misincorporation event. Depending on the cycle in which that error occurs (e.g., in an early replication cycle), the entire DNA amplified could contain the erroneously incorporated base, thus, giving rise to a mutated gene product.

There are several thermostable DNA polymerases known in the art which exhibit 3'-5' exonuclease activity, like B-type polymerases from thermophilic Archaeobacteria which are used for high fidelity DNA amplification. Thermostable polymerases exhibiting 3'-5' exonuclease activity may be isolated or cloned from *Pyrococcus* (Purified thermostable *Pyrococcus furiosus* DNA polymerase, Mathur E., Stratagene, WO 92/09689, US 5,545,552; Purified thermostable DNA polymerase from *Pyrococcus species*, Comb D. G. et al., New England Biolabs, Inc., EP 0 547 359; Organization and nucleotide sequence of the DNA polymerase gene from the archaeon *Pyrococcus furiosus*, Uemori T. et al. (1993) *Nucl. Acids Res.*, 21:259-265.), from *Pyrodictium spec.* (Thermostable nucleic acid polymerase, Gelfand D. H., F. Hoffmann-La Roche AG, EP 0 624 641; Purified thermostable nucleic acid polymerase and DNA coding sequences from *Pyrodictium species*, Gelfand D. H., Hoffmann-La Roche Inc., US 5,491,086), from *Thermococcus* (e.g. Thermostable DNA polymerase from *Thermococcus spec.* TY, Niehaus F., et al. WO 97/35988; Purified *Thermococcus barossii* DNA polymerase, Luhm R. A., Pharmacia Biotech, Inc., WO 96/22389; DNA polymerase from *Thermococcus barossii* with intermediate exonuclease activity and better long term stability at high temperature, useful for DNA sequencing, PCR etc., Dhennezel O. B., Pharmacia Biotech Inc., WO 96/22389; A purified thermostable DNA polymerase from *Thermococcus litoralis* for use in DNA manipulations, Comb D. G., New England Biolabs, Inc., US 5,322,785, EP 0 455 430; Recombinant thermostable DNA polymerase from Archaeobacteria, Comb D. G., New England Biolabs, Inc., US 5,352,778, EP 0 547 920, EP 0 701 000; New isolated thermostable DNA polymerase obtained from *Thermococcus gorgonarius*, Angerer B. et al. Boehringer Mannheim GmbH, WO 98/14590.

Another possibility of conferring PCR in the presence of a proofreading function is the use of a mixture of polymerase enzymes, one polymerase exhibiting such a proofreading activity. (e.g. Thermostable DNA polymerase with enhanced thermostability and enhanced length and efficiency of primer extension, Barnes W. M., US 5,436,149, EP 0 693 078; Novel polymerase

compositions and uses thereof, Sorge J. A., Stratagene, WO 95/16028). It is common practice to use a formulation of a thermostable DNA polymerase comprising a majority component of at least one thermostable DNA polymerase which lacks 3'-5' exonuclease activity and a minority component exhibiting 3'-5' exonuclease activity e.g. *Taq* polymerase and *Pfu* DNA polymerase. In these mixtures the processivity is conferred by the pol I-type enzyme like *Taq* polymerase, the proofreading function by the thermostable B-type polymerase like *Pfu*. High fidelity DNA synthesis is one desirable parameter in nucleic acid amplification, another important feature is the possibility of decontamination.

The polymerase chain reaction can amplify a single molecule over a billionfold. Thus, even minuscule amounts of a contaminant can be amplified and lead to a false positive result. Such contaminants are often products from previous PCR amplifications (carry-over contamination). Therefore, researchers have developed methods to avoid such a contamination.

The procedure relies on substituting dUTP for TTP during PCR amplification to produce uracil-containing DNA (U-DNA). Treating subsequent PCR reaction mixtures with Uracil-DNA-Glycosylase (UNG) prior to PCR amplification the contaminating nucleic acid is degraded and not suitable for amplification. dUTP can be readily incorporated by polI-type thermostable polymerases but not B-type polymerases (G. Slupphaug, et al. (1993) *Anal. Biochem.* 211:164-169) Low incorporation of dUTP by B-type polymerases limits their use in laboratories where the same type of template is repeatedly analyzed by PCR amplification.

Thermostable DNA polymerases exhibiting 3' - 5' exonuclease activity were also isolated from eubacterial strains like *Thermotoga* (Thermophilic DNA polymerases from *Thermotoga neapolitana*, Slater M. R. et al. Promega Corporation, WO 96/41014; Cloned DNA polymerases from *Thermotoga neapolitana* and mutants thereof, Hughes A. J. et al., Life Technologies, Inc. WO 96/10640; Purified thermostable nucleic acid polymerase enzyme from *Thermotoga maritima*, Gelfand D. H. et al., CETUS Corporation, WO 92/03556) These enzymes have a strong 3'-5' exonuclease activity which is able to eliminate misincorporated or mismatched bases. A genetically engineered version of this enzyme is commercially available as ULTma, a DNA polymerase which can be used without additional polypeptides for the PCR process. This enzyme is able to remove misincorporated bases, incorporate dUTP, but the fidelity is for unknown reasons not higher than that of *Taq* polymerase (Accuracy of replication in the polymerase chain reaction. Diaz R. S. et al. *Braz. J. Med. Biol. Res.* (1998) 31: 1239-1242; PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases, Cline J. et al., *Nucleic Acids Res.* (1996) 24:3546-3551).

For high fidelity DNA synthesis another alternative to the use of B-type polymerases or mixtures containing them is the use of thermophilic DNA polymerase III holoenzyme, a complex of 18 polypeptide chains. These complexes are identical to the bacterial chromosomal replicases, comprising all the factors necessary to synthesize a DNA strand of several hundred kilobases or whole chromosomes. The 10 different subunits of this enzyme, some of which are present in multiple copies, can be produced by recombinant techniques, reconstituted and used for *in vitro* DNA synthesis. As a possible use of these complexes PCR amplification of nucleic acids of several thousand to hundreds of thousand base pairs is proposed. (Enzyme derived from thermophilic organisms that functions as a chromosomal replicase, and preparation and uses thereof, Yurieva O. et al., The Rockefeller University, WO 98/45452; Novel thermophilic polymerase III holoenzyme, McHenry C., ENZYCO Inc., WO 99/13060)

It was aimed according to this invention to develop a high fidelity PCR system which is preferably concomitantly able to incorporate dUTP. According to the present invention a thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity is provided whereas this enzyme enhances fidelity of an amplification process when added to a second enzyme exhibiting polymerase activity. The enzyme provided can excise mismatched primer ends to allow the second enzyme exhibiting polymerase activity as e.g. Taq polymerase to reassociate and to resume elongation during a process of synthesizing DNA. The inventive enzyme is able to cooperate as proofreading enzyme with a second enzyme exhibiting polymerase activity. The enzyme that was found to be suitable for this task is e.g. a thermostable *exonuclease III*. Preferred is an *exonuclease III* working from the 3' to 5' direction, cleaving 5' of the phosphate leaving 3' hydroxyl groups and ideally working on double stranded DNA only. The 3'-5' exonuclease functions of DNA polymerases are active on double and single stranded DNA. The latter activity may lead to primer degradation, which is undesired in PCR assays. It is preferred that the enzyme is active at 70 °C to 80 °C, stable enough to survive the denaturation cycles and inactive at lower temperatures to leave the PCR products undegraded after completion of the PCR process. Enzymes exhibiting these features can be derived from thermophilic eubacteria or related enzymes from thermophilic archaea. Genomes of three thermostable archaeobacteria are sequenced, *Methanococcus jannaschii* (Complete Genome Sequence of the Methanogenic Archaeon, *Methanococcus jannaschii*, Bult C.J. et al., (1996) *Science* 273: 1058-1072), *Methanobacterium thermoautotrophicum* (Complete genomic sequence of *Methanobacterium thermoautotrophicum* ΔH: Functional Analysis and Comparative Genomics, Smith D.R. et al., *J. of Bacteriology*

(1997) 179: 7135-7155) and *Archaeoglobus fulgidus* (The complete genome sequence of the hyperthermophilic, sulfate-reducing archaeon *Archaeoglobus fulgidus*, Klenk H.-P. et al. (1997) *Nature* 390: 364-370).

In particular, there is provided a thermostable enzyme obtainable from *Archaeoglobus fulgidus*, which catalyzes the degradation of mismatched ends of primers or polynucleotides in the 3' to 5' direction in double stranded DNA. The gene encoding the thermostable exonuclease III obtainable from *Archaeoglobus fulgidus* (Afu) was cloned, expressed in *E.coli* and isolated. The enzyme is active under the incubation and temperature conditions used in PCR reactions. The enzyme supports DNA polymerases like *Taq* in performing DNA synthesis at low error rates and synthesis of products of more than 3 kb on genomic DNA – the upper range of products synthesized by *Taq* polymerase - in good yields with or without dUTP present in the reaction mixture. Preferably, 50-500 ng of the exonuclease III obtainable from Afu were used per 2,5 U of *Taq* polymerase in order to have an optimal PCR performance. More preferably is the use of 67 ng to 380 ng of the exonuclease III obtainable from Afu per 2,5 U of the *Taq* polymerase in the PCR reaction.

Thus, the inventive enzyme is able to cooperate as proofreading enzyme with *Taq* polymerase. The advantage of the use of the inventive enzyme in comparison to other enzymes is that the inventive enzyme is preferably active on double stranded DNA. The thermostable enzyme of this invention may be used for any purpose in which such enzyme activity is necessary or desired. In a particularly preferred embodiment the enzyme is used in combination with a thermostable DNA polymerase in the nucleic acid amplification reaction known as PCR in order to remove mismatched primer ends which lead to premature stops, to provide primer ends which are more effectively elongated by the polymerase, to correct for base incorporation errors and to enable the polymerase to produce long PCR products.

Further, subject of the present invention is a composition comprising a first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity and a second enzyme exhibiting polymerase activity whereas the fidelity of an amplification process is enhanced by the use of this composition in comparison to the use of the second enzyme alone. The inventive thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity also includes appropriate enzymes exhibiting reduced DNA polymerase

activity or no such activity at all. Reduced DNA polymerase activity according to the invention means less than 50% of said activity of an enzyme exhibiting DNA polymerase activity. In a preferred embodiment the second enzyme of the inventive composition is lacking proofreading activity. In particular preferred, the second enzyme is Taq polymerase.

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A further subject of the present invention is a method of DNA synthesis using a mixture comprising a first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity and a second enzyme exhibiting polymerase activity. According to this method prematurely terminated chains are trimmed by degradation from 3' to 5'. Mismatched ends of either a primer or the growing strand are removed according to this method.

The invention further comprises a method according to the above description whereas dUTP is present in the reaction mixture, replacing partly or completely TTP. It is preferred that according to this method uracil DNA glycosylase (UDG or UNG) is used for degradation of contaminating nucleic acids.

Preferably, according to this method the mixture of a

- first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity and
- a second enzyme exhibiting polymerase activity

produces PCR products with lower error rates compared to PCR products produced by the second enzyme exhibiting polymerase activity in absence of the first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity. The method in which the mixture of first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity and a second enzyme exhibiting polymerase activity produces PCR products of greater length compared to PCR products produced by the second enzyme exhibiting polymerase activity in absence of the first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity. Further, the first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity is related to the Exonuclease III of E. coli, but thermostable according to this method. A further embodiment of the above described method is the method whereas PCR products with blunt ends are obtained.

Subject of the present invention are also methods for obtaining the inventive thermostable enzyme exhibiting 3' exonuclease-activity but essentially no DNA polymerase activity and means

and materials for producing this enzyme as e.g. vectors and host cells (e.g. DSM no. 13021).

The following examples are offered for the purpose of illustrating, not limiting, the subject invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Brief description of the drawings

Figure 1:

DNA sequence and the deduced amino acid sequence of the gene encoding the DNA polymerase from exonuclease III of *Archaeoglobus fulgidus*.

Figure 2:

Resistance to heat denaturation of the recombinant exonuclease III of *Archaeoglobus fulgidus* expressed in *E.coli* as described in Example V.

Lane 1: Incubation at 50°C

Lane 2: Incubation at 60°C

Lane 3: Incubation at 70°C

Lane 4: Incubation at 80°C

Lane 5: Incubation at 90°C

Lane 6: *E.coli* host cell extract not transformed with gene encoding *Afu* exonuclease III

Lane 7: Exonuclease III of *E.coli*

Lane 8: Molecular weight marker

Figure 3:

Exonuclease activity of *Afu* exonuclease III on DNA fragments as described in Example VI.

Lane 1: 10 units *E.coli* exonuclease III, incubation at 37°C

Lane 2: 50 ng of *Afu* exonuclease III, incubation at 72°C

Lane 3: 100 ng of *Afu* exonuclease III, incubation at 72°C

Lane 4: 150 ng of *Afu* exonuclease III, incubation at 72°C

Lane 5: 100 ng of *Afu* exonuclease III, incubation at 72°C

Lane 6: 200 ng of *Afu* exonuclease III, incubation at 72°C

Lane 7: 300 ng of *Afu* exonuclease III, incubation at 72°C

Lane 8: 250 ng of *Afu* exonuclease III, incubation at 72°C

Lane 9: 750 ng of *Afu* exonuclease III, incubation at 72°C

Lane 10: 1 µg of *Afu* exonuclease III, incubation at 72°C

Lane 11: 500 ng of *Afu* exonuclease III, incubation at 72°C
Lane 12: 1 µg of *Afu* exonuclease III, incubation at 72°C
Lane 13: 1.5 µg of *Afu* exonuclease III, incubation at 72°C
Lane 14: 1.5 µg of *Afu* exonuclease III, incubation at 72°C
Lane 15: 3 µg of *Afu* exonuclease III, incubation at 72°C
Lane 16: 4.5 µg of *Afu* exonuclease III, incubation at 72°C
Lane 17: 7.6 µg of *Afu* exonuclease III, incubation at 72°C
Lane 18: 15.2 µg of *Afu* exonuclease III, incubation at 72°C
Lane 19: 22.8 µg of *Afu* exonuclease III, incubation at 72°C
Lane 20: no exonuclease added

Figure 4:

Principle of the mismatch correction assay.

Figure 5:

Mismatched primer correction in PCR as described in Example VII.

Lane 1: DNA Molecular Weight Marker V (ROCHE Molecular Biochemicals No. 821705)
Lane 2: G:A mismatched primer, amplification with *Taq* DNA polymerase
Lane 3: same as in lane 2, but subsequently cleaved with BsiEI
Lane 4: G:A mismatched primer, amplification with Expand HiFi PCR System
Lane 5: same as in lane 4, but subsequently cleaved with BsiEI
Lane 6: G:A mismatched primer, amplification with *Taq* polymerase/*Afu* exonuclease III
Lane 7: same as in lane 6, but subsequently cleaved with BsiEI
Lane 8: G:A mismatched primer, amplification with *Tgo* DNA polymerase
Lane 9: same as in lane 8, but subsequently cleaved with BsiEI
Lane 10: G:T mismatched primer, amplification with *Taq* DNA polymerase
Lane 11: same as in lane 10, but subsequently cleaved with BsiEI
Lane 12: G:T mismatched primer, amplification with Expand HiFi PCR System
Lane 13: same as in lane 12, but subsequently cleaved with BsiEI
Lane 14: G:T mismatched primer, amplification with *Taq* polymerase/*Afu* exonuclease III
Lane 15: same as in lane 14, but subsequently cleaved with BsiEI
Lane 16: G:T mismatched primer, amplification with *Tgo* DNA polymerase
Lane 17: same as in lane 16, but subsequently cleaved with BsiEI
Lane 18: DNA Molecular Weight Marker V
Lane 19: DNA Molecular Weight Marker V

Lane 20: G:C mismatched primer, amplification with *Taq* DNA polymerase
 Lane 21: same as in lane 20, but subsequently cleaved with BsiEI
 Lane 22: G:C mismatched primer, amplification with Expand HiFi PCR System
 Lane 23: same as in lane 22, but subsequently cleaved with BsiEI
 Lane 24: G:C mismatched primer, amplification with *Taq* polymerase/*Afu* exonuclease III
 Lane 25: same as in lane 24, but subsequently cleaved with BsiEI
 Lane 26: G:C mismatched primer, amplification with *Tgo* DNA polymerase
 Lane 27: same as in lane 26, but subsequently cleaved with BsiEI
 Lane 28: CG:AT mismatched primer, *Taq* DNA polymerase
 Lane 29: same as in lane 28, but subsequently cleaved with BsiEI
 Lane 30: CG:AT mismatched primer, Expand HiFi PCR System
 Lane 31: same as in lane 2, but subsequently cleaved with BsiEI
 Lane 32: CG:AT mismatched primer, *Taq* polymerase/*Afu* exonuclease III
 Lane 33: same as in lane 2, but subsequently cleaved with BsiEI
 Lane 34: CG:AT mismatched primer, amplification with *Tgo* DNA polymerase
 Lane 35: same as in lane 2, but subsequently cleaved with BsiEI
 Lane 36: DNA Molecular Weight Marker V.

Figure 6A:

Error rates of different polymerases in PCR

Figure 6B:

Improvement of fidelity by *Afu* exonuclease III present in the PCR mixture as described in Example VIII.

The ratio of blue:white colonies were blotted and various mixtures of *Taq* DNA polymerase and *Afu* exonuclease III (*Taq*/Exo 1:30, *Taq*/Exo 1:20, *Taq*/Exo 1:15, *Taq*/Exo 1:12,5, *Taq*/Exo 1:10 corresponding to 2.5 units of *Taq* DNA polymerase mixed with 125 ng, 175 ng, 250 ng, 375 ng and 500 ng of *Afu* exonuclease III, respectively) were tested in comparison to *Taq* DNA polymerase (*Taq*), Expand HiFi PCR System (HiFi) and *Pwo* DNA polymerase (*Pwo*).

Figure 7:

Incorporation of dUTP by the *Taq* DNA polymerase / *Afu* exonuclease III mixture as described in Example IX.

Lane 1: DNA Molecular Weight Marker XIV (Roche Molecular Biochemicals No. 1721933)
 Lane 2: Amplification with 2.5 units *Taq* DNA polymerase

- Lane 3: Amplification with 2.5 units *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III
 Lane 4: Amplification with 2.5 units *Taq* DNA polymerase and 250 ng of *Afu* exonuclease III
 Lane 5: Amplification with 2.5 units *Taq* DNA polymerase and 375 ng of *Afu* exonuclease III
 Lane 6: Amplification with 2.5 units *Taq* DNA polymerase and 500 ng of *Afu* exonuclease III

Figure 8:

Degradation of dUTP containing PCR products by Uracil-DNA Glycosylase as described in Example IX.

- Lane 1: DNA Molecular Weight Marker XIV (Roche Molecular Biochemicals No. 1721933)
 Lane 2: 1 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
 Lane 3: 2 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
 Lane 4: 3 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
 Lane 5: 4 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
 Lane 6: 5 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
 Lane 7: 5 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III no subsequent UNG or heat treatment.
 Lane 8: 5 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III no subsequent UNG but heat treatment.
 Lane 9: DNA Molecular Weight Marker XIV (Roche Molecular Biochemicals No. 1721933)

Figure 9:

Effect of *Afu* exonuclease III on PCR product length. The *Taq* DNA polymerase / *Afu* exonuclease III mixture was analyzed on human genomic DNA as described in Example X.

- Lane 1: 9,3 kb tPA fragment with *Taq*/Exo III Mix
 Lane 2: " " *Taq*-Pol.
 Lane 3: 12 kb tPA fragment with *Taq*/Exo III Mix
 Lane 4: " " *Taq*-Pol.

Lane 5: 15 kb tPA fragment with Taq/Exo III Mix

Lane 6: „ Taq-Pol.

Figure 10:

Thermostable exonuclease III can be replaced by a polymerase mutant with reduced polymerase activity but increased 3'-exonuclease-activity as described in Example XI.

Lane 1: Molecular Weight Marker

Lane 2: reaction 1, Taq polymerase, 4.8 kb fragment

Lane 3: reaction 2, Taq polymerase plus Tag polymerase mutant , 4.8 kb fragment

Lane 4: reaction 3, no Taq polymerase, Tag polymerase mutant , 4.8 kb fragment

Lane 5: reaction 4, Taq polymerase plus Afu ExoIII, 4.8 kb fragment

Lane 6: reaction 5, Taq polymerase, 9.3 kb fragment

Lane 7: reaction 6, Taq polymerase plus Tag polymerase mutant, 9.3 kb fragment Lane 8:

reaction 7, no Taq polymerase, Tag polymerase mutant , 9.3 kb fragment

Lane 9: reaction 8, Taq polymerase plus Afu ExoIII, 9.3 kb fragment

Lane 10: Molecular Weight Marker

Figure 11.

Afu exonuclease III is not active on linear single stranded DNA as described in Example XII

Lane 1: Afu Exo III, no incubation

Lane 2: Afu Exo III, 1 h at 65°C

Lane 3: Afu Exo III, 2 h at 65°C

Lane 4: Afu Exo III, 3 h at 65°C

Lane 5: Afu Exo III, 4 h at 65°C

Lane 6: Afu Exo III, 5 h at 65°C

Lane 7: Reaction buffer without enzyme, no incubation

Lane 8: Reaction buffer without enzyme, 5 h at 65°C

Lane 9: Molecular Weight Marker

Figure 12:

Comparison of Afu exonuclease III with a thermostable B-type polymerase in primer degrading activity as described in Example XIII.

- Lane 1: Molecular Weight Marker
- Lane 2: 1 u Tgo preincubated (reaction 1)
- Lane 3: 1.5 u Tgo, preincubated (reaction 2)
- Lane 4: 1 u Tgo, not preincubated (reaction 3)
- Lane 5: 1.5 u Tgo, not preincubated (reaction 4)
- Lane 6: 1 u Tgo, preincubated in the absence of dNTPs (reaction 5)
- Lane 7: 1.5 u Tgo, preincubated in the absence of dNTPs (reaction 6)
- Lane 8: 1 u Tgo, not preincubated in the absence of dNTPs (reaction 7)
- Lane 9: 1.5 u Tgo, not preincubated in the absence of dNTPs (reaction 8)
- Lane 10: 1 u Tgo, preincubated, in the absence of dNTPs, supplemented with additional primer (reaction 9)
- Lane 11: 1.5 u Tgo, preincubated in the absence of dNTPs, supplemented with additional primer (reaction 10)
- Lane 12: Taq polymerase, preincubated (reaction 11)
- Lane 13: Taq plus 37,5 ng Afu Exo III, preincubated (reaction 12)
- Lane 14: Taq plus 75 ng Afu Exo III, preincubated (reaction 13)
- Lane 15: Taq polymerase, not preincubated (reaction 14)
- Lane 16: Taq plus 37,5 ng Afu Exo III, not preincubated (reaction 15)
- Lane 17: Taq plus 75 ng Afu Exo III, not preincubated (reaction 16)
- Lane 18: Molecular Weight Marker

EXAMPLE I

Isolation of coding sequences

The preferred thermostable enzyme herein is an extremely thermostable exodeoxyribonuclease obtainable from *Archaeoglobus fulgidus* VC-16 strain (DSM No. 4304). The strain was isolated from marine hydrothermal systems at Vulcano island and Stufe di Nerone, Naples, Italy (Stetter, K. O. et al., *Science* (1987) 236:822-824). This organism is an extremely thermophilic, sulfur metabolizing, archaebacteria, with a growth range between 60°C and 95°C with optimum at 83°C. (Klenk, H.P. et al., *Nature* (1997) 390:364-370). The genome sequence is deposited in the TIGR data base. The gene putatively encoding exonuclease III (xthA) has Acc.No. AF0580.

The apparent molecular weight of the exodeoxyribonuclease obtainable from *Archaeoglobus fulgidus* is about 32,000 daltons when compared with protein standards of known molecular weight (SDS-PAGE). The exact molecular weight of the thermostable enzyme of the present invention may be determined from the coding sequence of the *Archaeoglobus fulgidus* exodeoxyribonuclease III gene.

EXAMPLE II

Cloning of the gene encoding exonuclease III from *Archaeoglobus fulgidus*

About 6 ml cell culture of DSM No. 4304 were used for isolation of chromosomal DNA from *Archaeoglobus fulgidus*.

The following primers were designed with restriction sites compatible to the multiple cloning site of the desired expression vector and complementary to the N- and C-terminus of the *Archaeoglobus fulgidus* exonuclease III gene:

SEQ ID NO.: 1

N-terminus (BamHI-site): 5'-GAA ACG AGG ATC CAT GCT CAA AAT CGC CAC C-3'

SEQ ID NO.: 2

C-terminus (PstI-site): 5'-TTG TTC ACT GCA GCT ACA CGT CAA ACA CAG C-3'

First the cells were collected by repeated centrifugation in one 2 ml eppendorf cap at 5,000 rpm. The DNA isolation may be performed with any described method for isolation from bacterial cells. In this case the *Archaeoglobus fulgidus* genomic DNA was prepared with the High Pure™ PCR Template Preparation Kit (ROCHE Diagnostics GmbH, No. 1796828). With this method about 6 µg chromosomal DNA were obtained with a concentration of 72 ng/µl.

PCR was performed with the primers described above, in the Expand™ High Fidelity PCR System (ROCHE Diagnostics GmbH, No. 1732641) and 100 ng *Archaeoglobus fulgidus* genomic DNA per cap in four identical preparations. PCR was performed with the following conditions:

- 1 x 94°C, 2 min;
- 10 x 94°C, 10 sec; 54°C, 30 sec; 68°C, 3 min;

- 20 x 94°C, 10 sec; 54°C, 30 sec; 68°C, 3 min with 20sec cycle elongation for each cycle;
1 x 68°C, 7 min;

After adding $MgCl_2$ to a final concentration of 10 mM the PCR product was cleaved with BamHI and Pst I, 10 units each, at 37°C for 2 hours. The reaction products were separated on a low-melting agarose gel. After electrophoresis the appropriate bands were cut out, the gel slices combined, molten, the DNA fragments isolated by agarase digestion and precipitated with EtOH. The dried pellet was diluted in 30 μ l H_2O .

The appropriate expression vector, here pDS56_T, was digested with the same restriction enzymes as used for the insert and cleaned with the same method.

After ligation of insert and vector with the Rapid DNA Ligation Kit (ROCHE Diagnostics GmbH, No.1635379) the plasmid was transformed in the expression host *E.coli* 392 pUBS520 (Brinkmann, U. et al. (1989) *Gene* 85:109-114).

Plasmid DNA of the transformants was isolated using the High Pure™ Plasmid Isolation Kit (ROCHE Diagnostics GmbH, No.1754777) and characterized by restriction digestion with BamHI and PstI and agarose gel electrophoresis.

Positive *E.coli* pUBS520 ExoIII transformants were stored in glycerol culture at -70°C. The sequence of the gene encoding exonuclease III was confirmed by DNA sequencing. It is shown in Figure No. 1.

Cloning and expression of exonuclease III from *Archaeoglobus fulgidus* or other thermophilic organisms may also be performed by other techniques using conventional skill in the art (see for example Sambrook et al. Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Lab., 1989).

EXAMPLE III

Expression of recombinant *Afu* exonuclease III

The transformant from example I was cultivated in a fermentor in a rich medium containing appropriate antibiotic. Cells were harvested at an optical density of $[A_{540}]$ 5.5 by centrifugation

and frozen until needed or lyzed by treatment with lysozyme to produce a crude cell extract containing the *Archaeoglobus fulgidus* exonuclease III activity.

The crude extract containing the *Archaeoglobus fulgidus* exonuclease III activity is purified by the method described in example IV, or by other purification techniques such as affinity-chromatography, ion-exchange-chromatography or hydrophobic-interaction-chromatography.

EXAMPLE IV

Purification of recombinant *Afu* exonuclease III

E.coli pUBS520 ExoIII (DSM No. 13021) from example I was grown in a 10 l fermentor in media containing tryptone (20 g/l), yeast extract (10 g/l), NaCl (5 g/l) and ampicillin (100 mg/l) at 37°C, induced with IPTG (0.3 mM) at midexponential growth phase and incubated an additional 4 hours. About 45 g of cells were harvested by centrifugation and stored at - 70°C. 2 g of cells were thawed and suspended in 4 ml buffer A (40 mM Tris/HCl, pH 7.5; 0.1 mM EDTA; 7 mM 2-mercaptoethanol; 1mM Pefabloc SC). The cells were lyzed under stirring by addition of 1.2 mg lysozyme for 30 minutes at 4°C and addition of 4.56 mg sodium deoxycholate for 10 minutes at room temperature followed by 20 minutes at 0°C. The crude extract was adjusted to 750 mM KCl, heated for 15 minutes at 72°C and centrifuged for removal of denatured protein.

A heating temperature up to 90 °C is also possible without destroying (denaturation) the *Archaeoglobus fulgidus* exonuclease III. The supernatant was dialyzed against buffer B (buffer A containig 10 % glycerol) adjusted to 10 mM MgCl₂ and applied to a Blue Trisacryl M column (SERVA, No. 67031) with the dimension 1 x 7 cm and 5.5 ml bed volume, equilibrated with buffer B. The column was washed with 16.5 ml buffer B and the exonuclease protein was eluted with a 82 ml linear gradient of 0 to 3 M NaCl in buffer B. The column fractions were assayed for *Archaeoglobus fulgidus* exodeoxyribonuclease protein by electrophoresis on 10-15% SDS-PAGE gradient gels. The active fractions, 16.5 ml, were pooled, concentrated with Aquacide II (Calbiochem No. 17851) and dialyzed against the storage buffer C (10 mM Tris/HCl, pH 7.9; 10 mM 2-mercpctoethanol; 0.1mM EDTA; 50 mM KCl; 50 % glycerol). After dialysis Thesit and Nonidet P40 were added to a final concentration of 0.5% each. This preparation was stored at - 20 °C.

The *Archaeoglobus fulgidus* exonuclease III obtained was pure to 95% as estimated by SDS gel electrophoresis. The yield was 50 mg of protein per 2.3g cellmass (wetweight).

EXAMPLE V

Thermostability of recombinant exonuclease III from *Archaeoglobus fulgidus*

The thermostability of the exonuclease III from *Archaeoglobus fulgidus* cloned as described in Example II was determined by analyzing the resistance to heat denaturation. After lysis as described in Example IV 100 µl of the crude extract were centrifuged at 15,000 rpm for 10 min in an Eppendorf centrifuge. The supernatant was aliquoted into five new Eppendorf caps. The caps were incubated for 10 minutes at five different temperatures, 50°C, 60°C, 70°C, 80°C and 90°C. After centrifugation as described above, aliquotes of the supernatants were analyzed by electrophoresis on 10-15 % SDS-PAGE gradient gels. As shown in Figure 2 the amount of *Archaeoglobus fulgidus* exonuclease III protein after incubation at 90°C was the same as that of the samples treated at lower temperatures. There was no significant loss by heat denaturation detectable. From this result it can be concluded that the half life is more than ten minutes at 90°C.

EXAMPLE VI

Activity of *Afu* exonuclease III

Exonuclease III catalyzes the stepwise removal of mononucleotides from 3'-hydroxyl termini of duplex DNA (Rogers G.S. and Weiss B. (1980) *Methods Enzymol.* 65:201-211). A limited number of nucleotides are removed during each binding event. The preferred substrates are blunt or recessed 3'-termini. The enzyme is not active on single stranded DNA, and 3'-protruding termini are more resistant to cleavage. The DNA Molecular Weight Marker VI (ROCHE Molecular Biochemicals, No.1062590) consists of BglI digested pBR328 mixed with HinfI digested pBR328. The products of the HinfI digest have 3'-recessive termini and are expected to be preferred substrates to degradation by exonuclease III, the products of BglI cleavage have 3' protruding ends with 3 bases overhangs and should be more resistant to cleavage by exonuclease III.

Serial dilutions of *Archaeoglobus fulgidus* exonuclease III from Example IV were incubated for 2 hours at 72 °C with 0.5 µg DNA Molecular Weight Marker VI (ROCHE Molecular Biochemicals, No.1062590) in 25 µl of the following incubation buffer: 10 mM Tris/HCl, pH 8.0; 5 mM MgCl₂; 1 mM 2-mercaptoethanol; 100 mM NaCl with Paraffin overlay. 10 units of exonuclease III of *E.coli* (ROCHE Molecular Biochemicals, No.779709) was included as a control. The control

reaction was performed at 37°C. After addition of 5 µl stop solution (0.2 % Agarose, 60 mM EDTA, 10 mM Tris-HCl, pH 7.8, 10 % Glycerol, 0.01 % Bromphenolblue) the mixtures were separated on a 1 % agarose gel. The result is shown in Figure 3. *Afu* exonuclease III discriminates between the two different types of substrate. The preferred substrate are the fragments with 3'-recessive ends (e.g. 1766 bp fragment) and the 3'-overhanging ends (e.g. 2176 bp, 1230bp, 1033 bp fragments) are more resistant to degradation. With higher amounts of protein the substrate is degraded to a similar extent as in lane 1, where the products of exonuclease III of *E.coli* were analyzed. With increasing amounts of *Afu* exonuclease protein only little DNA substrate was left (lanes 15 to 19), the retardation of the remaining fragments may be due to DNA binding proteins as impurities of the preparation.

EXAMPLE VII

Mismatched primer correction in PCR with *Afu* exonuclease III

The repair efficiency of the *Afu* exonuclease III / *Taq* polymerase mixture during PCR was tested with 3' terminally mismatched primers, the principle of the assay is shown in Figure 4. For PCR amplification sets of primers are used in which the forward primer has one or two nucleotides at the 3' end which cannot base pair with the template DNA. Excision of the mismatched primer end and amplification of the repaired primer generates a product which can subsequently be cleaved with the restriction endonuclease BsiEI, whereas the product arising from the mismatched primer is resistant to cleavage.

The primer sequences used :

1. reverse: 5' - GGT TAT CGA AAT CAG CCA CAG CG - 3'
(SEQ ID NO.: 3)
2. forward 1 (g:a mismatch): 5' - TGG ATA CGT CTG AAC TGG TCA CGG TCA - 3'
(SEQ ID NO.: 4)
3. forward 2 (g:t mismatch): 5' - TGG ATA CGT CTG AAC TGG TCA CGG TCT - 3'
(SEQ ID NO.: 5)
4. forward 3 (g:c mismatch): 5' - TGG ATA CGT CTG AAC TGG TCA CGG TCC - 3'
(SEQ ID NO.: 6)
5. forward 4 (2 base mismatch): 5' - TGG ATA CGT CTG AAC TGG TCA CGG TAT - 3'
(SEQ ID NO.: 7)

PCR was carried out using 2.5 Units *Taq* DNA Polymerase (ROCHE Diagnostics GmbH, No. 1435094), 0.25 µg of *Archaeoglobus fulgidus* exonuclease III from Example IV, 10 ng of DNA from bacteriophage λ, 0.4 µM of each primer, 200 µM of dNTP's, 1.5 mM of MgCl₂, 50 mM of Tris-HCl, pH 9.2, 16 mM of (NH₄)₂SO₄. PCR was performed in a volume of 50 µl PCR with the following conditions:

- 1 x 94°C, 2 min;
- 40 x 94°C, 10 sec; 60°C, 30 sec; 72°C, 1 min;
- 1 x 72°C, 7 min;

The function of the exonuclease/*Taq* polymerase mixture was compared to controls as 2.5 Units of *Taq* DNA polymerase, 0.3 Units of *Tgo* DNA polymerase (ROCHE Diagnostics GmbH) and to 0.75 µl of Expand™ High Fidelity PCR System (ROCHE Diagnostics GmbH, No.1732641). As indicated by successful digestion of the PCR products with BsiEI *A. fulgidus* exonuclease III showed correcting activity of all described mismatches with an effectivity of 90 to 100 % (Figure 5). *Taq* DNA Polymerase as expected showed no correcting activity, while *Tgo* DNA Polymerase with its 3'-5' exonuclease activity corrected completely as well. The Expand™ High Fidelity PCR System showed only with the two base mismatch 100% correcting activity. The other mismatches were repaired with an effectivity of approximately 50%.

EXAMPLE VIII

Fidelity of *Afu* exonuclease III /*Taq* DNA polymerase mixtures in the PCR process

The fidelity of *Afu* exonuclease III/*Taq* DNA polymerase mixtures in the PCR process was determined in an assay based on the amplification, circularisation and transformation of the pUC19 derivative pUCIQ17, containing a functional *lac* I^q allele (Frey, B. and Suppmann B. (1995) *Biochemica* 2:34-35). PCR-derived mutations in *lac* I are resulting in a derepression of the expression of *lac* Zα and subsequent formation of a functional β-galactosidase enzyme which can be easily detected on X-Gal indicator plates. The error rates of *Taq* polymerase /*Afu* exonuclease mixtures determined with this *lac* I-based PCR fidelity assay were determined in comparison to *Taq* DNA polymerase and Expand HiFi PCR System (Roche Molecular Biochemicals) and *Pwo* DNA polymerase (Roche Molecular Biochemicals) as controls.

The plasmid pUCIQ17 was linearized by digestion with DraII to serve as a substrate for PCR amplification with the enzymes tested.

Both of the primers used have ClaI sites at their 5 prime ends:

SEQ ID NO.: 8

Primer 1: 5'-AGCTTATCGATGGCACTTTTCGGGGAAATGTGCG-3'

SEQ ID NO.: 9

Primer 2: 5'-AGCTTATCGATAAGCGGATGCCGGGAGCAGACAAGC-3'

The length of the resulting PCR product is 3493 bp.

The PCR was performed in a final volume of 50 μ l in the presence of 1.5 mM MgCl₂, 50 mM Tris HCl, pH 8.5 (25°C), 12.5 mM (NH₄)₂SO₄, 35 mM KCl, 200 μ M dNTPs and 2.5 units of *Taq* polymerase and 125 ng, 175 ng, 250 ng, 375 ng and 500 ng, respectively of *Afu* exonuclease III.

The cycle conditions were as follows:

1 x denaturation of template for 2 min. at 95°C

8 x $\left\{ \begin{array}{l} \text{denaturation at 95°C for 10 sec.} \\ \text{annealing at 57°C for 30 sec.} \\ \text{elongation at 72°C for 4 min.} \end{array} \right.$

16 x $\left\{ \begin{array}{l} \text{denaturation at 95°C for 10 sec.} \\ \text{annealing at 57°C for 30 sec.} \\ \text{elongation at 72°C for 4 min.} \\ \text{+ cycle elongation of 20 sec. for each cycle} \end{array} \right.$

After PCR, the PCR products were PEG-precipitated (Barnes, W. M. (1992) *Gene* 112:229) the DNA restricted with ClaI and purified by agarose gel electrophoresis. The isolated DNA was ligated using the Rapid DNA Ligation Kit (Roche Molecular Biochemicals) and the ligation products transformed in *E.coli* DH5 α , plated on TN Amp X-Gal plates. The α -complementing *E.coli* strain DH5 α transformed with the resulting plasmid pUCIQ17 (3632 bp), shows white (lacI⁺) colonies on TN plates (1.5 % Bacto Tryptone, 1 % NaCl, 1.5 % Agar) containing ampicillin (100 μ g/ml) and X-Gal (0.004 % w/v). Mutations result in blue colonies.

After incubation overnight at 37°C, blue and white colonies were counted. The error rate (f) per bp was calculated with a rearranged equation as published by Keohavong and Thilly (Keohavong, P. and Thilly, W. (1989) *PNAS USA* 86:9253):

$$f = -\ln F / d \times b \text{ bp}$$

where F is the fraction of white colonies:

$$F = \text{white (lacI+)} \text{ colonies} / \text{total colony number};$$

d is the number of DNA duplications:

$$2^d = \text{output DNA} / \text{input DNA};$$

and b is the effective target size of the (1080bp) *lac I* gene, which is 349 bp according to Provost et al. (Provost et al. (1993) *Mut. Res.* 288:133).

The results shown in Figure 6A and Figure 6B demonstrate that the presence of thermostable exonuclease III in the reaction mixture results in lower error rates. Dependent on the ratio of polymerase to exonuclease the error rate is decreasing. The fidelity achieved with the most optimal *Taq* polymerase / *Afu* exonuclease III mixture ($4,44 \times 10^{-6}$) is in a similar range as that of the *Taq/Pwo* mixture (Expand HiFi; $2,06 \times 10^{-6}$). Evaluation of the optimal buffer conditions will further improve the fidelity. The ratio between polymerase and exonuclease has to be optimized. High amounts of exonuclease reduce product yield, apparently decreasing amplification efficiency (*Taq/Exo* 1:10 corresponding to 2.5 units of *Taq* polymerase and 500 ng of *Afu* exonuclease III).

The fidelity of this system may further be optimized using conventional skill in the art e.g. by altering the buffer components, optimizing the concentration of the individual components or changing the cycle conditions.

EXAMPLE IX:

Incorporation of dUTP in the presence of *Afu* exonuclease III during PCR

The *Afu* exonuclease/*Taq* polymerase mixture was tested for DNA synthesis with TTP completely replaced by dUTP. Comparison of either TTP or dUTP incorporation was determined in PCR using 2.5 Units of *Taq* DNA Polymerase, in presence of 0.125 µg, 0.25 µg, 0.375 µg and 0.5 µg of *Archaeoglobus fulgidus* exonuclease III from example IV on native human genomic DNA as template using the β -globin gene as target. The following primers were used:

forward: 5' - TGG TTG AAT TCA TAT ATC TTA GAG GGA GGG C - 3'
(SEQ ID NO.: 10)
reverse: 5' - TGT GTC TGC AGA AAA CAT CAA GGG TCC CAT A - 3'
(SEQ ID NO.: 11)

PCR was performed in 50 µl volume with the following cycle conditions:

1	x	94°C, 2 min;
40	x	94°C, 10 sec; 60°C, 30 sec; 72°C, 1 min;
1	x	72°C, 7 min;

Aliquots of the PCR reaction were separated on agarose gels. As shown in Figure 7 with this template/primer system DNA synthesis in the presence of dUTP is possible with up to 375 ng of *Afu* exonuclease III. dUTP incorporation can further be proven by Uracil-DNA Glycosylase treatment (ROCHE Diagnostics GmbH, No.1775367) of aliquotes from the PCR reaction products for 30 min at ambient temperature and subsequent incubation for 5 min at 95°C to cleave the polynucleotides at the apurinic sites which leads to complete degradation of the fragments. The analysis of the reaction products by agarose gel electrophoresis is shown in Figure 8.

EXAMPLE X:

Effect of *Afu* exonuclease III on PCR product length

Taq polymerase is able to synthesize PCR products up to 3 kb in length on genomic templates. In order to estimate the capability of the *Taq* polymerase/*Afu* exonuclease mixture for the synthesis of longer products, the enzyme mixture was analyzed on human genomic DNA as template with

three pairs of primers designed to amplify products of 9.3 kb, 12 kb and 15 kb length. The buffer systems used were from the Expand Long Template PCR System (Roche Molecular Biochemicals Cat. No 1 681 834). Reactions were performed in 50 µl volume with 250 ng of human genomic DNA, 220 ng of each primer, 350 µM of dNTPs and 2.5 units of *Taq* polymerase and 62,5 ng of *Afu* exonuclease with the conditions as outlined in Table 1:

Table 1:

Product length	Primers	Expand Long Template buffer No.:	PCR Programm
9.3 kb	forward 7 reverse 14	1	1 x denat. at 94 °C for 2 min
			10 x denat. at 94°C for 10 sec. annealing at 65°C for 30 sec elongation at 68°C for 8 min.
			20 x denat. at 94°C for 10 sec. annealing at 65°C for 30 sec elongation at 68°C for 8 min. plus cycle elongation of 20 sec. per cycle
12 kb	forward 1 reverse 3	2	1 x elongation at 68°C for 7 min.
			1 x denat. at 94 °C for 2 min
			10 x denat. at 94°C for 10 sec. annealing at 62°C for 30 sec elongation at 68°C for 12 min.
			20 x denat. at 94°C for 10 sec. annealing at 62°C for 30 sec elongation at 68°C for 12 min. plus cycle elongation of 20 sec. per cycle
15 kb	forward 1 reverse 2	3	1 x elongation at 68°C for 7 min.
			same as for 12 kb

The primer specific for amplification of the tPA genes used:

Primer 7a forward: 5' - GGA AGT ACA GCT CAG AGT TCT GCA GCA CCC CTG C - 3'
(SEQ ID NO.: 12)

Primer 14a reverse: 5' - CAA AGT CAT GCG GCC ATC GTT CAG ACA CAC C - 3'
(SEQ ID NO.: 13)

Primer 1 forward: 5' - CCT TCA CTG TCT GCC TAA CTC CTT CGT GTG TCC C - 3'
(SEQ ID NO.: 14)

Primer 2 reverse: 5' - ACT GTG CTT CCT GAC CCA TGG CAG AAG CGC CTT C - 3'
(SEQ ID NO.: 15)

Primer 3 reverse: 5' - CCT TCT AGA GTC AAC TCT AGA TGT GGA CTT AGA G - 3'
(SEQ ID NO.: 16)

As shown in Figure 9 it is possible to synthesize products of at least 15 kb in length with the *Taq* polymerase/*Afu* exonuclease mixture.

Example XI

Thermostable Exonuclease III can be replaced by a polymerase mutant with reduced polymerase activity but increased 3' exonuclease-activity

DNA polymerase from *Thermococcus aggregans* (Tag) described from Niehaus F., Frey B. and Antranikian G. in WO97/35988 or *Gene* (1997) 204 (1-2), 153-8, with an amino acid exchange at position 385 in which tyrosine was replaced by asparagine (Boehlke et al. submitted for publication and European patent application 00105 155.6) shows only 6.4 % of the polymerase activity but 205 % of the exonuclease activity of the wild type DNA polymerase. This enzyme was used to demonstrate that the invention is not restricted to exonuclease III-type enzymes but also includes other types of enzymes contributing 3' exonuclease activity.

Reactions were performed in 50 µl volume with 200 ng of human genomic DNA, 200 µM dNTP, 220 ng of each primer and Expand HiFi buffer incl. Mg⁺⁺ for reactions 1-4 or Expand Long Template buffer 1 for reactions 5-8 (Figure 10). In order to amplify a 4.8 kb fragment of the tPA gene, primer tPA 7a forward (5'-GGA AGT ACA GCT CAG AGT TCT GCA GCA CCC CTG C-3', SEQ ID NO.: 12) and tPA 10a reverse (5'-GAT GCG AAA CTG AGG CTG GCT GTA CTG TCT C-3', SEQ ID NO.: 17) were used in reactions 1 - 4. In order to amplify a 9.3 kb fragment of the tPA gene, primer tPA 7a forward and tPA 14a reverse (5'-CAA AGT CAT GCG GCC ATC GTT CAG ACA CAC C-3', SEQ ID NO.: 13) were used in reactions 5-8. 2.5 units *Taq* polymerase were added to reactions 1,2,4,5,6, and 8, not to reactions 3 and 7 which were used as negative controls. 11 ng of Tag polymerase mutant were added to reactions 2,3, 6 and 7, 150 ng of *Afu* Exonuclease III were added to reactions 4 and 8.

The cycle programs used for reactions 1-4:

1 x	94°C, 2 min,
10 x	94°C, 10 sec
	62°C, 30 sec
	68°C, 4 min
20 x	94°C, 10 sec
	62°C, 30 sec

68°C, 4 min, plus cycle elongation of 20 sec per cycle

1x 68°C for 7 min

for reactions 5-8:

1 x 94°C, 2 min,

10 x 94°C, 10 sec

65°C, 30 sec

68°C, 8 min

20 x 94°C, 10 sec

65°C, 30 sec

68°C, 8 min, plus cycle elongation of 20 sec per cycle

1x 68°C for 7 min

The PCR products were analysed on a 1 % agarose gel containing ethidium bromide (Figure 10). The data show that Taq polymerase is able to amplify the 4.8 kb fragment but with low yield. The combination of Taq polymerase with Tag polymerase mutant or Afu Exo III results in a strong increase in product yield. The Tag polymerase mutant enzyme by itself is not able to synthesize this product.

Similar results were obtained with the 9.3 kb system. Using Taq polymerase alone no product is detectable. In combination with Tag polymerase mutant or Afu Exo III the expected PCR product is obtained in high yield.

These results show that Taq polymerase is not able to amplify DNA fragments of several kb from genomic DNA and support the hypothesis of Barnes (Barnes W. M. (1994) *Proc. Natl. Acad. Sci. USA*, 91:2216-2220) that the length limitation for PCR amplification is caused by low efficiency of extension at the sites of incorporation of mismatched base pairs. After removal of the mismatched nucleotide at the primer end, Taq polymerase is able to reassume DNA synthesis. The completed nucleic acid chain as a full length product can then serve as a template for primer binding in subsequent cycles.

Example XII

Afu Exo III is not active on linear single stranded DNA

Reactions were performed in 50 µl volume with 270 ng of Afu Exo III, 5 µg of a 49-mer oligonucleotide in Expand HiFi PCR buffer with MgCl₂ and incubated for 0, 1, 2, 3, 4, and 5 hours at 65°C. After addition of 10 µl of Proteinase K solution (20 mg/ml) the samples were incubated for 20 min. at 37°C. The reaction products were analysed on a 3.5 % Agarose gel containing ethidium bromide.

The result is depicted in figure 11. It shows that the nucleic acid has the same size in all lanes. The product obtained after incubation for up to 5 hours (lane 6) with Afu Exo III has the same size as the controls (lanes 1, 7 and 8). Neither a significant reduction in intensity of the full length oligonucleotide nor a smear deriving from degraded products can be observed.

Example XIII

Comparison of Afu Exonuclease III with a thermostable B-type polymerase in primer degrading activity

Thermostable B-type polymerases are reported to have single and double stranded nuclease activity (Kong H. et al. (1993) *Journal Biol. Chem.* 268:1965-1975). This activity is able to degrade primer molecules irrespective whether they are hybridized to the template or single stranded. The replacement of a thermostable B-type polymerase by a thermostable exonuclease in the reaction mixture might be of advantage with respect to stability of single stranded primer or other nucleic acids present in the reaction mixture.

In order to test for primer degrading activity, reaction mixtures without template DNA were incubated for 1 hour at 72°C, then DNA was added and PCR was performed. The results were compared with reactions containing Tgo polymerase as an example for a thermostable B-type polymerase (Angerer B. et al. WO 98/14590). As control the same mixtures were used without prior incubation. The results are summarized in Table 2.

Table 2:

reaction #	enzyme (s)	preincubation in the absence of template DNA	preinc. in the presence of nucleotides	second addition of primer after preincubaion
1	Tgo	yes	yes	
2	Tgo	yes	yes	
3	Tgo	no		
4	Tgo	no		
5	Tgo	yes	no	
6	Tgo	yes	no	
7	Tgo	no		
8	Tgo	no		
9	Tgo	yes	no	yes
10	Tgo	yes	no	yes
11	Taq	yes	yes	
12	Taq plus Afu Exo III	yes	yes	
13	Taq plus Afu Exo III	yes	yes	
14	Taq	no		
15	Taq plus Afu Exo III	no		
16	Taq plus Afu Exo III	no		

As target for amplification a fragment of the p53 gene was chosen, the primer used were: p53I 5'-GTC CCA AGC AAT GGA TGA T-3' (SEQ ID NO.: 18) and p53II 5'-TGG AAA CTT TCC ACT TGA T-3' (SEQ ID NO.: 19). PCR reactions were performed in 50 μ l volume.

Reactions nos. 1 – 10 contained 200 ng of human genomic DNA, 40 pmole of each primer, 10 mM Tris-HCl, pH 8.5, 17.5 mM $(\text{NH}_4)_2\text{SO}_4$, 1.25 mM MgCl_2 , 0.5 % Tween, 2.5 % DMSO, 250 μ g/ml BSA and 1 unit (reactions number 1, 3, 5, 7 and 9) or 1.5 units (reactions number 2, 4, 6, 8 and 10) Tgo polymerase and 200 μ M dNTPs.

Reactions number 11 to 16 contained 2.5 units Taq polymerase, Expand HiFi buffer with Mg^{++} , 40 pmoles of primer, 200 μ M dNTPs, 100 ng human genomic DNA. Reactions number 12 and 15 contained 37.5 ng of Afu Exo III, reactions number 13 and 16 contained 75 ng of Afu Exo III.

As described in table 2 reactions 1, 2, 5, 6 and 11 to 13 were incubated for 1 hour at 72°C in the absence of template DNA. The template DNA was added before PCR was started. Reactions 5, 6, 9 and 10 were preincubated in the absence of nucleotides, reactions 9 and 10 were supplemented with additional 40 pmoles of primer after the preincubation step. Because of the 5'- exonuclease activity of Taq polymerase, the enzyme was added after preincubation to reactions 11 to 13.

PCR conditions:

1 x 94°C, 2 min

35 x 94°C, 10 sec

55°C, 30 sec

72°C, 4 min

1x 72°C for 10 min

The reaction products were analysed on an agarose gel and stained with ethidium bromide (Figure 12).

When Tgo polymerase was incubated with the primer in the absence of template DNA (reactions 1,2,5 and 6) and compared with the corresponding reactions without preincubation (3,4,7 and 8) a clear difference was observed. The preincubation results in strongly reduced PCR product obviously affecting at least one essential component, most probably the PCR primer. Extra addition of 40 pmoles of PCR primer (reactions 9 and 10) after the preincubation step results in strong signals with intensities comparable to the control reaction which were not preincubated. This shows that Tgo polymerase, a thermostable B-type polymerase, degrades PCR primer in the absence of template no matter whether dNTPs are present or not.

The PCR products obtained with reactions 12 and 13, in which the primer were preincubated with Afu Exonuclease III before addition of template DNA and Taq polymerase gave similar bands as those obtained with reactions 15 and 16, in which no preincubation step was used. From the similar strong band intensities it can be concluded that little or no degradation of primer occurred and that single stranded oligonucleotides are poor substrates for Afu Exonuclease III. From the strong band intensities or enhanced yields of PCR products it can be concluded that the enzyme enhances fidelity of an amplification process.